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Award Number: DAMD17-02-1-0626

TITLE: Ack-1 Tyrosine Kinase Regulates Integrin Signaling
Leading to Breast Cell Migration

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REPORT DATE: March 2003

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

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REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE March 2003	3. REPORT TYPE AND DATES COVERED Annual Summary (1 Mar 02 - 28 Feb 03)	
4. TITLE AND SUBTITLE Ack-1 Tyrosine Kinase Regulates Integrin Signaling Leading to Breast Cell Migration		5. FUNDING NUMBERS DAMD17-02-1-0626	
6. AUTHOR(S): Katarzyna Modzelewska Patricia J. Keely, Ph.D.			
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) University of Wisconsin-Madison Madison, Wisconsin 53706-1490 E-Mail: kmodzelewska@wisc.edu		8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012		10. SPONSORING / MONITORING AGENCY REPORT NUMBER	
11. SUPPLEMENTARY NOTES		20030724 023	
12a. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited		12b. DISTRIBUTION CODE	
13. Abstract (Maximum 200 Words) (abstract should contain no proprietary or confidential information) Appropriate interactions between breast cells and the ECM via $\alpha 2\beta 1$ integrin help to establish normal cellular structure and differentiation. During transformation to a carcinoma, these normal interactions with the ECM are profoundly altered, resulting in cells that lose their specialization and lose control of their growth. Ultimately, these cells become invasive, and then migrate through the connective tissue environment to form distant metastases. We have previously found that Ack-1 tyrosine kinase enhances $\alpha 2\beta 1$ integrin-induced cell migration and regulates signaling components downstream of the integrin. In the course of the research I have determined that Ack-1 is phosphorylated upon collagen stimulation and that this phosphorylation is dependent on Src and FAK kinases. Furthermore, I found that FAK is not required for Ack-1 association with p130Cas or Src and that Src is not necessary for p130Cas and FAK association with Ack-1. I also determined that Ack-1 associates with the SH3 domains of p130Cas, Src and Spectrin, which suggest direct binding between these molecules.			
14. SUBJECT TERMS: Ack-1, non-receptor tyrosine kinase, integrin, Rho family GTPases, signaling		15. NUMBER OF PAGES 9	
		16. PRICE CODE	
17. SECURITY CLASSIFICATION OF REPORT Unclassified	18. SECURITY CLASSIFICATION OF THIS PAGE Unclassified	19. SECURITY CLASSIFICATION OF ABSTRACT Unclassified	20. LIMITATION OF ABSTRACT Unlimited

Table of Contents

Cover.....	1
SF 298.....	2
Introduction.....	4
Body.....	5-7
Key Research Accomplishments.....	8
Reportable Outcomes.....	8
Conclusions.....	9
References.....	9

Introduction

Appropriate interactions between breast cells and the ECM via $\alpha 2\beta 1$ integrin help to establish normal cellular structure and differentiation. During transformation to a carcinoma, these normal interactions with the ECM are profoundly altered, resulting in cells that lose their specialization and lose control of their growth. Ultimately, these cells become invasive, and then migrate through the connective tissue environment to form distant metastases (1,2). We have previously found that Ack-1 tyrosine kinase enhances $\alpha 2\beta 1$ integrin-induced cell migration and regulates signaling components downstream of the integrin. The purpose of this research is to understand the mechanism by which Ack-1 is phosphorylated and how the kinase regulates integrin-dependent signaling leading to cell migration. To do that I will investigate the role of FAK and Src in Ack-1 phosphorylation, as well as screen for Ack-1 binding partners and determine whether these interactions are collagen dependent and whether they play a role in cell migration.

Specific Aims and Research Accomplishments

1) Determine how Ack-1 is phosphorylated upon collagen stimulation and whether this phosphorylation is important in cell migration.

Rationale

Our preliminary results suggested that Ack-1 is phosphorylated upon activation of $\alpha 2\beta 1$ integrin but the mechanism by which this occurs is unknown. It has been established that integrin stimulation leads to activation of FAK and Src. Because Ack-1 participates in a complex with FAK and Src (Figure 6), it may be regulated by FAK or Src in a collagen-dependent manner. I will test whether this is accomplished via direct phosphorylation by FAK or by FAK activation of Src, which in turn phosphorylates Ack-1. I will also investigate the importance of Ack-1 phosphorylation in collagen-induced migration.

Research Accomplishments

Thus far I have determined that the phosphorylation of Ack-1 is dependent on both FAK and Src kinases.

To determine the role of Src kinase in Ack-1 phosphorylation control and Cdc42(12V) expressing T47D cells treated with the specific Src kinase inhibitor, PP2 exhibited reduced Ack-1 phosphorylation (Figure 1). Cells were pretreated with the PP2 inhibitor, or DMSO for 15 minutes and then stimulated with collagen or control buffer for 10 minutes in suspension and lysed. Ack-1 was immunoprecipitated and its phosphorylation was determined by immunoblotting with the anti-phosphotyrosine antibody. Both control and Cdc42(12V) expressing cells treated with PP2 showed reduced levels of phosphorylation suggesting that Src plays a role in phosphorylating Ack-1.

My second approach was to utilize fibroblasts from Src(-/-) mice (purchased from ATCC). Src knockout fibroblasts (SYF) and control (SYF cells re-expressing cSrc) cells, were pretreated with DMSO or PP2 inhibitor for 15 minutes, and subsequently stimulated with collagen for 10 minutes in suspension and lysed. Ack-1 was immunoprecipitated and levels of its phosphorylation were determined by immunoblotting with anti-phosphotyrosine antibody.

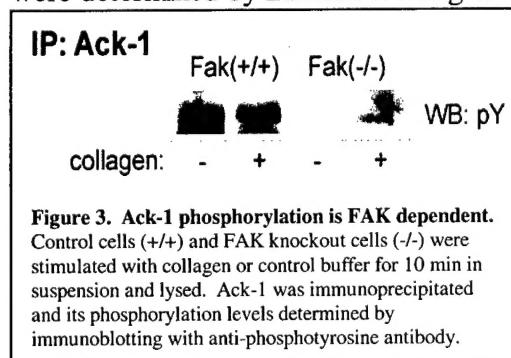


Figure 3. Ack-1 phosphorylation is FAK dependent. Control cells (+/+) and FAK knockout cells (-/-) were stimulated with collagen or control buffer for 10 min in suspension and lysed. Ack-1 was immunoprecipitated and its phosphorylation levels determined by immunoblotting with anti-phosphotyrosine antibody.

fibroblasts (ATCC). Control cells (normal fibroblasts) and FAK knockout cells were stimulated with

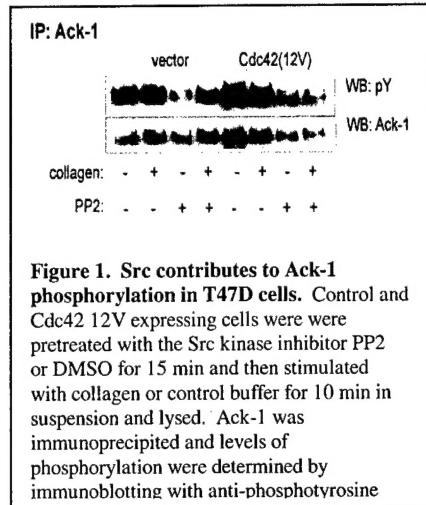


Figure 1. Src contributes to Ack-1 phosphorylation in T47D cells. Control and Cdc42(12V) expressing cells were pretreated with the Src kinase inhibitor PP2 or DMSO for 15 min and then stimulated with collagen or control buffer for 10 min in suspension and lysed. Ack-1 was immunoprecipitated and levels of phosphorylation were determined by immunoblotting with anti-phosphotyrosine

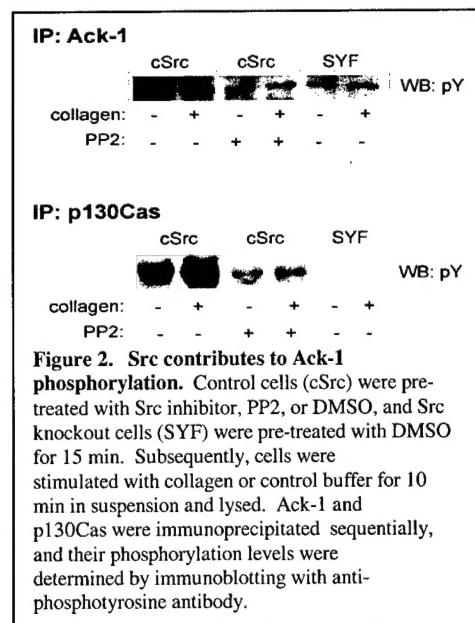


Figure 2. Src contributes to Ack-1 phosphorylation. Control cells (cSrc) were pretreated with Src inhibitor, PP2, or DMSO, and Src knockout cells (SYF) were pre-treated with DMSO for 15 min. Subsequently, cells were stimulated with collagen or control buffer for 10 min in suspension and lysed. Ack-1 and p130Cas were immunoprecipitated sequentially, and their phosphorylation levels were determined by immunoblotting with anti-phosphotyrosine antibody.

Control cells pretreated with PP2 and SYF cells exhibited reduced Ack-1 phosphorylation (Figure 2). As a control, p130Cas was immunoprecipitated sequentially and its phosphorylation was also determined. p130Cas phosphorylation is entirely lost in the SYF cells and almost fully abrogated in control cells pre-treated with PP2 (Figure 2). These results suggest that Src kinase activity is important but not solely responsible for Ack-1 phosphorylation.

I also investigated whether Focal Adhesion Kinase (FAK) plays a role in phosphorylating Ack-1. To do that I used FAK(-/-)

collagen or control buffer for 10 min in suspension and lysed. Ack-1 was immunoprecipitated from cell lysates and its phosphorylation was determined by immunoblotting with anti-phosphotyrosine antibody. Ack-1 phosphorylation was almost entirely lost in FAK knockout cells (Figure 3). In the next set of experiments I will re-express wild type and kinase dead FAK in the FAK(-/-) cells to determine whether kinase activity of FAK is crucial to Ack-1 phosphorylation. Once I establish that FAK activity is important I will proceed with the kinase assay to determine whether Ack-1 is a direct substrate for FAK.

2) Define the molecular interactions of Ack-1 and establish their importance in Cdc42-dependent cell migration.

Rationale

In addition to its kinase domain Ack-1 contains a CRIB domain, which is responsible for its interaction with Cdc42 (3), an SH3 domain, and a C-terminus rich in prolines. SH3 domains are known to interact with proline rich motifs. Thus far there have not been any molecules shown to interact with the Ack-1 SH3 domain. The proline rich region of Ack-1 interacts with clathrin, and is thought to bind to Src kinase (4,5). I will screen libraries of SH3 domains and proline rich domains to identify potential Ack-1 interacting partners. During this stage of my project, I will collaborate with the laboratory of Dr. Brian Kay who is an expert in combinatorial chemistry and specifically protein-protein interactions.

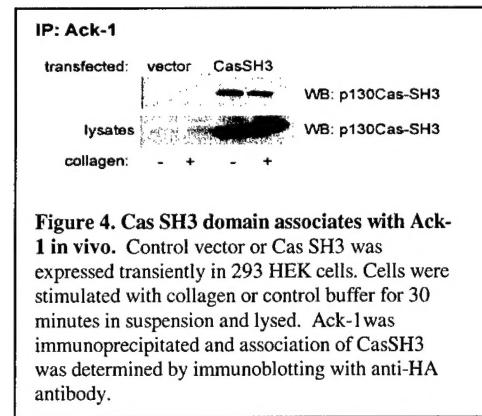


Figure 4. Cas SH3 domain associates with Ack-1 in vivo. Control vector or Cas SH3 was expressed transiently in 293 HEK cells. Cells were stimulated with collagen or control buffer for 30 minutes in suspension and lysed. Ack-1 was immunoprecipitated and association of CasSH3 was determined by immunoblotting with anti-HA antibody.

Research Accomplishments

I have determined that p130Cas, Src, and spectrin SH3 domains associate with Ack-1.

I have learned the phage display technique at the laboratory of Dr. Brian Kay, Argonne National Labs, and performed an initial screen with the Ack-1 SH3 domain.

I have previously shown that p130Cas associated with Ack-1 in a collagen dependent manner. I have now determined that p130CasSH3 domain co-immunoprecipitates with Ack-1 (Figure 4). Control vector or p130Cas SH3 was expressed transiently in 293 HEK cells. Cells were stimulated with collagen or control buffer for 30 minutes in suspension and lysed. Ack-1 was immunoprecipitated and association of p130CasSH3 was determined by immunoblotting with anti-HA antibody. The SH3 domain of p130Cas associates with Ack-1 independently of collagen stimulation. To further investigate the binding between p130Cas and Ack-1 via the SH3 domain of p130Cas, I subcloned the p130CasSH3 into a GST expression vector, and I am in the process of performing GST pull downs. In addition, I will express an SH3 deletion mutant of p130Cas in HEK293 cells and determine whether it still can bind to Ack-1. Ideally, I would like to show a direct interaction between the p130Cas SH3 domain and the Ack-1 proline rich region in an *in vitro* binding

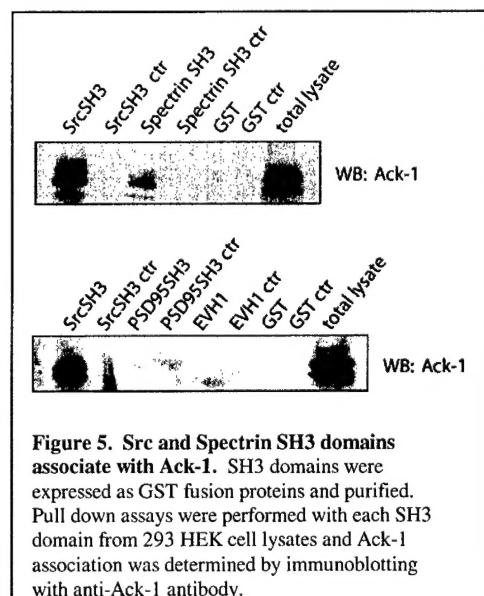


Figure 5. Src and Spectrin SH3 domains associate with Ack-1. SH3 domains were expressed as GST fusion proteins and purified. Pull down assays were performed with each SH3 domain from 293 HEK cell lysates and Ack-1 association was determined by immunoblotting with anti-Ack-1 antibody.

assay. However, I have had some trouble expressing the proline rich region fused to GST in bacteria. I am in the process of trying different bacterial strains and induction protocols.

I also determined that the SH3 domains of Src and Spectrin associate with Ack-1 in GST pull down assay (Figure 5). A panel of GST fusion SH3 domains as well as an EVH1 domain (which also binds proline rich motifs) were expressed as GST fusion proteins and purified from bacteria. Pull down assays were performed with each domain from 293 HEK cell lysates and Ack-1 association was determined by immunoblotting with anti-Ack-1 antibody. Src and spectrin SH3 domains were able to associate with Ack-1. In addition Src, associates with Ack-1 in cells in a collagen independent manner (Figure 6). I have not yet begun addressing the significance of these interactions in cell migration.

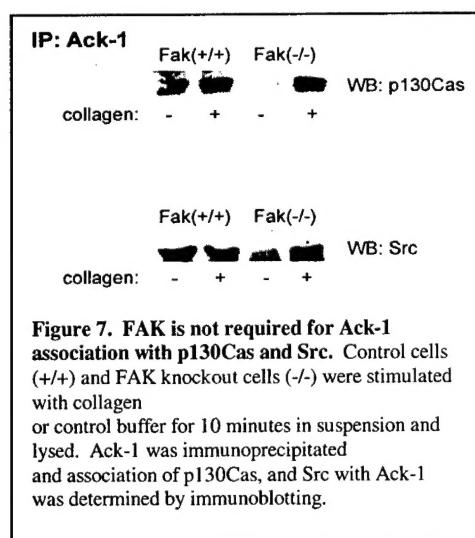


Figure 7. FAK is not required for Ack-1 association with p130Cas and Src. Control cells (+/+) and FAK knockout cells (-/-) were stimulated with collagen or control buffer for 10 minutes in suspension and lysed. Ack-1 was immunoprecipitated and association of p130Cas, and Src with Ack-1 was determined by immunoblotting.

In addition, I took advantage of the knockout cells at hand and looked whether Ack-1 association with its interacting partners depends on Src and FAK. Control cells (cSrc or FAK(+/+)) and Src or FAK knockout cells were stimulated with collagen or control buffer for 10 minutes in suspension and lysed. Ack-1 was immunoprecipitated and association of p130Cas, FAK, and Src with Ack-1 was determined by immunoblotting.

The results demonstrate that Src is not necessary for the association of Ack-1 with p130Cas or FAK (Figure 6) and that FAK is not required for the association of Src and p130Cas with Ack-1 (Figure 7). This data further suggests that the interactions of FAK, p130Cas and Src with Ack-1 are direct.

Finally, in addition to my original proposal I took advantage of the yeast two hybrid facility on campus and submitted the proline rich region of Ack-1 for a screen against a mouse library. I have not yet received any result from the screen.

3) Determine whether p130Cas is a substrate for Ack-1 and identify whether additional binding partners of Ack-1 are substrates for the kinase.

Rationale

Our preliminary data suggests that Ack-1 interacts with p130Cas and also that p130Cas is phosphorylated in a collagen-dependent manner. The substrate domain of p130Cas is composed of 15 YXXP motifs that are potential phosphorylation sites. I will test whether p130Cas substrate domain serves as a substrate for the Ack-1 kinase. I will also investigate whether any other binding partner of Ack-1 identified during the library screens is a substrate for Ack-1 kinase.

I have not begun addressing this aim.

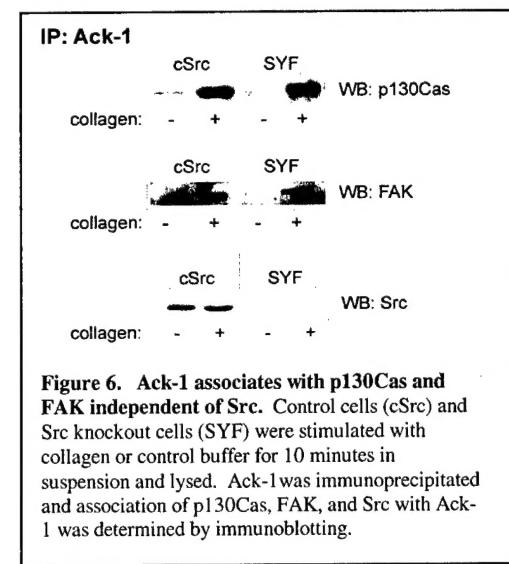


Figure 6. Ack-1 associates with p130Cas and FAK independent of Src. Control cells (cSrc) and Src knockout cells (SYF) were stimulated with collagen or control buffer for 10 minutes in suspension and lysed. Ack-1 was immunoprecipitated and association of p130Cas, FAK, and Src with Ack-1 was determined by immunoblotting.

Key Research Accomplishments

- Found that FAK and Src kinases contribute to phosphorylation of Ack-1
- Established that Src is not required for Ack-1 binding to p130Cas and FAK
- Established that FAK is not necessary for Ack-1 association with p130Cas and Src
- Determined that Src, Spectrin and p130Cas SH3 domains interact with Ack-1
- Learned phage display and performed an initial screen
- Manuscript in revision: **“Ack-1 mediates Cdc42-dependent signaling to p130Cas downstream of integrins”**
- Manuscript in preparation: **“Src and FAK regulate Ack-1 tyrosine kinase phosphorylation but are not required for downstream complex formation”**

Reportable Outcomes

Poster presentation

3rd International Conference on Signal Transduction

17-23 May, 2002

Dubrovnik, Croatia

Abstract

Src and FAK regulate Ack-1 tyrosine kinase phosphorylation but are not required for its downstream signaling

It has been established that Cdc42 induces cell migration through integrins. We have found that the Cdc42 effector - Ack-1 (Cdc42 associated kinase) regulates migration of T47D breast carcinoma cells across collagen through the $\alpha 2\beta 1$ integrin. To investigate the role of Ack-1 tyrosine kinase in cell migration, we looked at Ack-1 phosphorylation and presumably activation, and also its association with other signaling molecules involved in cell migration, such as focal adhesion kinase (FAK), Crk associated substrate (p130Cas) and Src kinase. Ack-1 phosphorylation was increased upon collagen stimulation. Surprisingly, this event was not regulated by the Cdc42 activation, since activated Cdc42 (12V) did not enhance Ack-1 phosphorylation and the dominant negative Cdc42 (17N) did not block it. We found, however, that Ack-1 phosphorylation was regulated by both Src and FAK kinases. Src kinase inhibitor PP2 significantly decreased Ack-1 phosphorylation. Ack-1 phosphorylation was also minimal in the SYF cells (Src, Yes, Fyn $-/-$) as compared to SYF cells over-expressing c-Src. Phosphorylation of Ack-1 was also diminished in the FAK $-/-$ cells, which suggests a role for the kinase in Ack-1 activation. Ack-1 could be co-immunoprecipitated with both FAK and Src, as well as with p130Cas. FAK and p130Cas association with Ack-1 is minimal in the absence of collagen and increases significantly upon $\alpha 2\beta 1$ integrin stimulation. This is in contrast to the association of Ack-1 with Src, which was not regulated by collagen. Interestingly, Ack-1 association with Src, FAK and p130Cas appears to be independent of the Cdc42 activation state. Furthermore, we find that the interactions between Ack-1 and p130Cas or Ack-1 and FAK do not require Src, since they can be co-immunoprecipitated in the SYF cells. Also, FAK is not necessary for the association between Ack-1 and p130Cas or Src. Finally, we established that Ack-1 plays a role in collagen-stimulated phosphorylation of FAK and p130Cas, since kinase dead Ack-1 blocked p130Cas and enhanced FAK phosphorylation. Our results suggest that Src and FAK regulate Ack-1 phosphorylation but not Ack-1 interactions with its signaling partners, and that Ack-1 contributes to the regulation of FAK and p130Cas phosphorylation downstream of the $\alpha 2\beta 1$ integrin.

Conclusions

I have found that Ack-1 phosphorylation is diminished in both Src and FAK knockout cells. However, neither Src or FAK are required for Ack-1 binding to the known interacting partners. In addition, I determined that p130Cas, Src and Spectrin SH3 domains associate with Ack-1, which implies direct binding. These results suggest that Src and FAK regulate Ack-1 phosphorylation but not Ack-1 interactions with its signaling partners downstream of the $\alpha 2\beta 1$ integrin.

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